Properties of the Disorder Induced in the Purple Membrane Structure by Iodination

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Abstract: Iodination induces disorder in the crystalline structure of purple membrane. The properties of the disorder were examined by X-ray diffraction experiments on the iodinated purple membrane. The analysis of the intensity and the integral width of the Bragg reflections revealed that the disorder was well characterized by the second kind of disorder. The g value, which represents the extent of the second kind of disorder, is obtained for iodinated purple membrane with various extent of iodination. The g value and the wavelength at absorption maximum of iodinated PM are well correlated, indicating that the local change around retinal is reflected in the disorder in the crystalline structure of purple membrane.

Key words: Purple membrane, Disorder, Chemical modification, Iodination, X-ray diffraction, Absorption spectra

1. Introduction

Purple membrane (PM) is a specifically differentiated part of the cell membrane of Halobacterium halobium; it contains only one protein, bacteriorhodopsin (bR) [1,2]. In the PM, bR molecules aggregate in a trimer to form a two-dimensional crystal [3]. Because the important physiological function of bR, is light-driven proton pumping, many investigations have been performed on the function and the structure of bR and PM [1]. As for the structure, since PM is a two-dimensional crystalline array of bR molecules, various diffraction methods were

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effectively applied to reveal the structure [3-11]. At present, a low resolution (3.5Å) structure is available [10] and the position of the retinal has also been deduced [11,12].

Apart from its physiological function, PM is an excellent example of a two dimensional crystal. Two dimensional crystals are known to exhibit different features from normal three dimensional crystals [13]. Therefore, PM is of interest from the view point of crystal physics. As for the physical properties of PM as a two dimensional crystal, a thermal phase transition has been widely investigated [14-17] and gave information on the structural stability of bR [15].

We have found that the iodination induces disorder in the PM structure [18]. This indicates that the physical state of iodinated PM is somewhat different from the native state. Iodinated PM shows characteristic properties as for the stability of the M intermediate other than its remarkable spectroscopic features [19-21]. It is interesting to study the relationship between these properties and the physical state of iodinated PM. The detailed analysis of the disorder will reveal the physical state of iodinated PM. The properties of disorder can be roughly derived from the analysis of the line width and the intensity of Bragg reflection.

We have performed small angle X-ray diffraction experiments on iodinated PM using synchrotron radiation and analyzed the induced disorder. As the result, the disorder induced by iodination can be analyzed in terms of the second kind of disorder. The shift of the absorption maximum around 560nm due to iodination is closely related to the growth of the second kind of disorder. Further we speculate that M intermediate is stabilized by the second kind of disorder.

2. Materials and Methods

PM was prepared using standard methods from H. halobium (R1m1) [22]. Iodination of PM was performed enzymatically using lactoperoxidase and glucose oxidase, as described previously [18]. In order to obtain PM with various extents of iodination the reaction time was varied from 10 min to 8h or the concentration of NaI was varied. The extent of iodination was monitored spectroscopically by measuring the absorbance at 560nm and the wavelength of maximum absorbance with a Shimadzu double beam spectrophotometer UV 300S. Iodinated PM was resuspended in 10mM phosphate buffer (pH 7.2).

X-ray diffraction experiments were performed with synchrotron radiation (SR) by a so-called Muscle Diffractometer [23,24] installed at BL15A line of the Photon Factory.

Iodinated PM suspended in 10mM phosphate buffer (pH 7.2, A_{560nm} =90) was introduced in the specially designed specimen cell with a window of

10mm width, 5 mm height and 1 mm path. The diffraction pattern was recorded with a position sensitive proportional counter with an effective length of 200 mm. Specimen to detector distance was from 60 to 100 cm and calibrated with Bragg reflection of native PM. The exposure time was 10 min at room temperature. The intensity of incident X-rays was monitored by an ionization chamber placed in front of the specimen. The data were transferred to a MINC 11/23 minicomputer, stored on a floppy disk and processed with an ACOS 1000 computer at Tohoku University Computer Center.

3. Results

3.1 Properties of the disorder induced in the iodinated PM

As reported previously [18], iodination induces some disorder into purple membrane crystalline structure, which is clear from X-ray diffraction patterns (Fig. 1). They are corresponding to the PM with 0, 1,

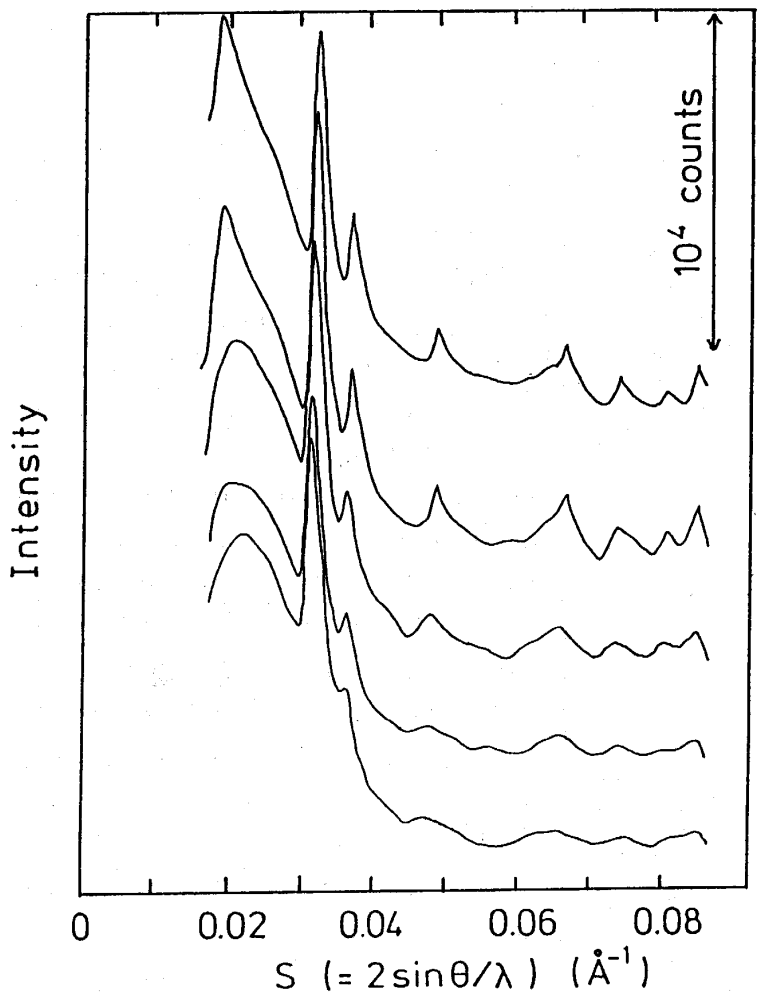

Fig. 1 X-ray diffraction patterns of iodinated PM's. From top to bottom: native PM; 20 min-iodinated PM; 2 h-iodinated PM; 4 h-iodinated PM; 8 h-iodinated PM. Blurring of the higher reflection peaks is clearly observed.

4, 6 and more than 6 iodine atoms per one molecule of bR [18]. In terms of the crystal physics, disorder induced in the crystalline structure is classified into two categories: the first kind and the second kind [25,26]. The first kind of disorder is due to thermal oscillations around the lattice point or substitution disorder in alloys. The second kind one is known by an ideal paracrystal. Liquid is also considered the limiting case of the second kind of disorder. These disorders are discriminated by the properties of the diffraction pattern. For the former case, the expected change in diffraction pattern is a decrease of the intensity of the Bragg reflection. For the latter case, broadening of the reflection widths is expected as well as a decrease of the intensities. Moreover, the broadening of the reflection width depends on the scattering angle. Therefore, the property of the disorder firstly can be deduced from the reflection line width. We utilized the integral width for the reflection line width, which was calculated by dividing the integral intensity by peak height of each reflection.

In Fig. 2, the integral width of each Bragg reflection was plotted against its reciprocal distance (scattering angle). In the figure, we can

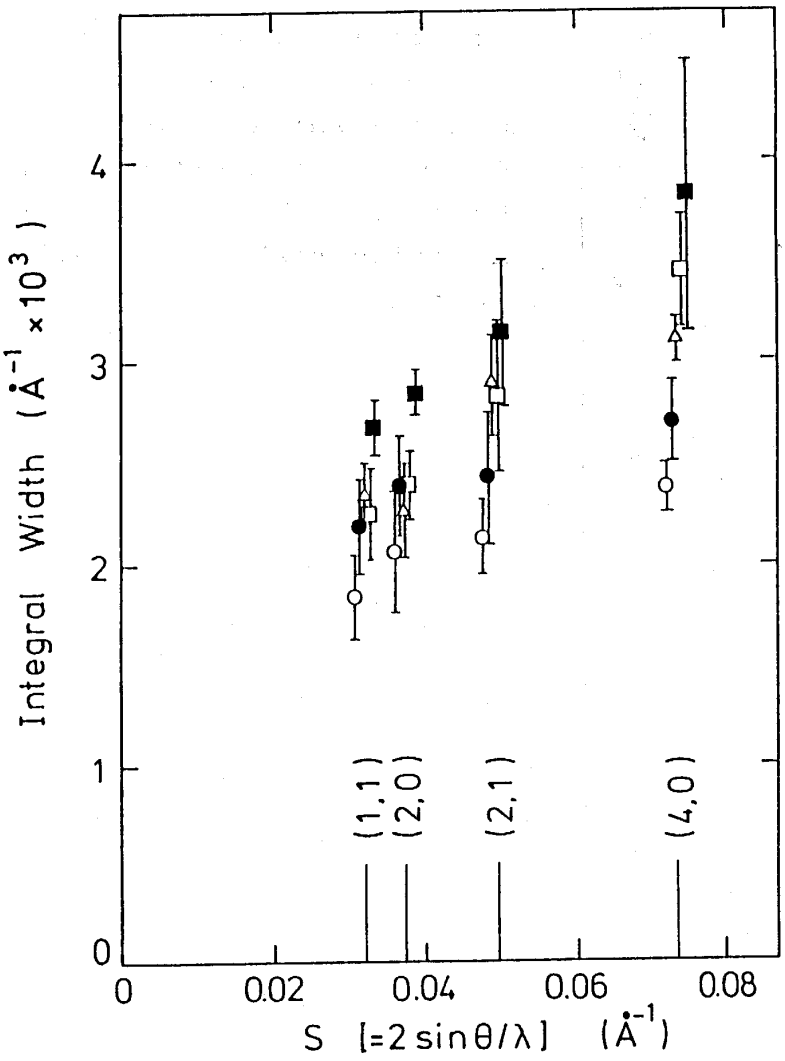

Fig. 2 Dependence of the integral width on the reciprocal distance S. S is defined as $2\sin\theta/\lambda$, where 2θ is scattering angle, λ is X-ray wavelength (1.5Å). The extent of iodination was controlled by the reaction time. The reaction mixture contained 40 M bR and 0.53 mM of NaI. ○; native PM, ●; 1 h iodinated, △; 2 h iodinated, □; 4 h iodinated and ■; 8 h iodinated PM. The average of five series of experiments are shown. Data for the same reflection are slightly shifted each other for the sake of clarity.

find that the integral widths for the iodinated PM's are broader than those for the native PM. As for the fixed reflection, there is a tendency that the width is broader for the PM with a longer iodination time. In fact, this tendency was clearly confirmed, when the widths were compared among one series of experiments. Moreover, the dependence of integral widths on scattering angle was clearly observed for 4h and 8h iodinated PM. These facts indicate that the second kind of disorder is induced in the PM structure by iodination, and is especially remarkable for 4h and 8h iodinated samples which bears 6 and more than 6 iodine atoms per one bR respectively.

The widths for native PM are mainly due to the geometry of the optics and spatial average for two dimensional system, and are not due to disorder. Broadening due to such origins are commonly observed for every specimen, and mathematically, these origins are convoluted to each reflection. Therefore, we can say that the above qualitative discussion is reasonable.

Disorder is also reflected in the intensities of the Bragg reflections. Figure 3 shows an example of the change in relative intensities of Bragg reflections against iodination time obtained from one series of experiments. In order to obtain Fig. 3, the ratio of the Bragg reflection intensity of iodinated PM to corresponding reflection intensity of native PM is defined as the relative intensity. Clearly, the intensities decrease as iodination proceeds for three Bragg reflections. We stress here that not only the iodinated PM but also the native one essentially contains the first kind of disorder as follows. The fact that the native PM in the dark-adapted state is an equimolar mixture of bR with all trans retinal and one with 13-cis retinal suggests the substitution disorder in the native PM, because the crystallographic symmetry of the PM is P3 and also Stamatoff et al. showed that the structural difference between the light-adapted PM and the dark-adapted PM [27]. In the early stage of iodination, some bR's are iodinated and others are not. The distribution of iodinated bR and non-iodinated one is expected to be random, indicating the substitution disorder (the first kind of disorder). Similar situation is realized at every stage of iodination. Therefore, the decrease of the intensity of each Bragg reflection should be explained partly by the first kind of disorder.

3.2 Quantitative analysis of the second kind of disorder

The analysis of the first kind of disorder is performed by measuring the diffuse scattering which appears around each Bragg reflection or between Bragg reflections [25,26]. In order to measure diffuse scattering, background scattering which is generated from sources other

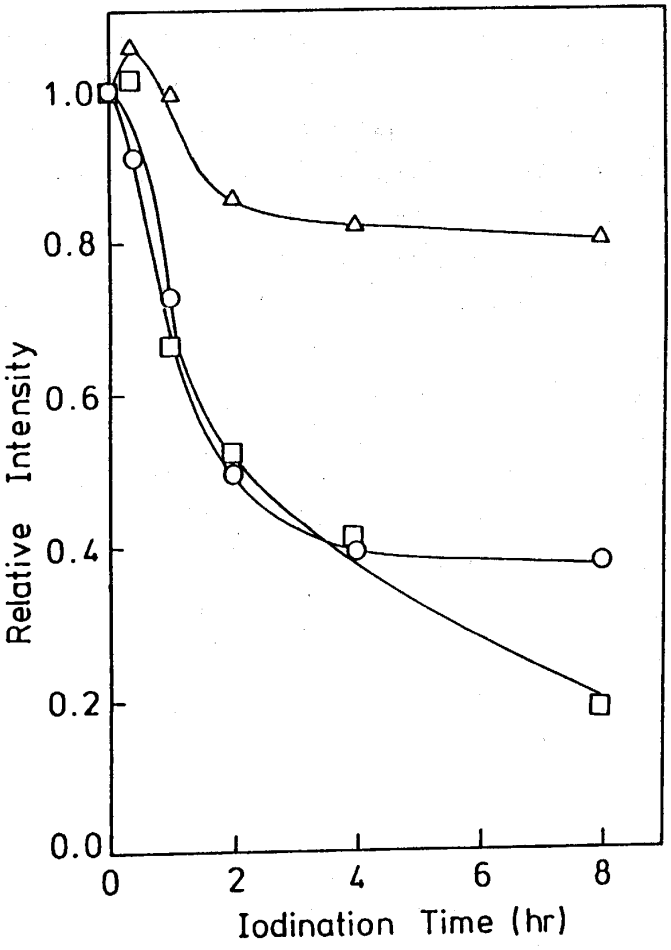

Fig. 3 The changes of relative intensities against iodination time. The ratio of the intensity of each Bragg reflection of iodinated PM to native PM was plotted against iodination time. △; (11) reflection, ○; (20) reflection and □; (40) reflection, respectively. The reaction conditions were the same as in Fig. 2, but obtained from just one of five series of experiments.

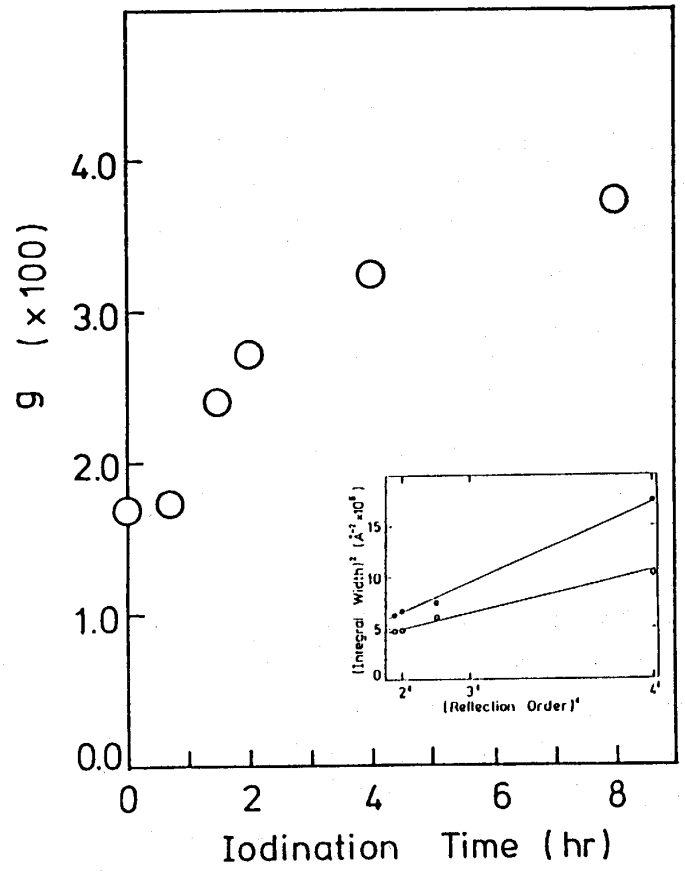

Fig. 4 The change of g value against iodination time. The g value represents the extent of the second kind of disorder. The disorder became prominent beyond 1 h of iodination time. Inset, the square of the integral width was plotted against the 4 th power of reflection order $\sqrt{h^2 + k^2 + hk}$. ○, for 4h iodinated PM and ●, for 8h iodinated PM respectively. From the slope, the g value can be calculated.

than crystalline structure of PM should be exactly subtracted from the diffraction data. Some portion of background scattering can be subtracted experimentally. However, there are many sources of background scattering which cannot be experimentally or theoretically estimated, such as membrane thickness and deviation from flatness of PM. The ambiguity for background subtraction makes the quantitative analysis of the first kind of disorder impossible.

On the other hand, the second kind of disorder can be analyzed from the line width of Bragg reflection [25,26]. The essential point for the discussion of the second kind of disorder is allowance for the short range order [25,26], that is, the mean distance a between nearest neighbor lattice point which is equal to the lattice constant can be defined. Also the distribution function H(x) at the nearest neighbor which defines the probability of finding a first neighbor is considered. Using H(x), a is calculated as $a = \int xH(x)dx$. The extent of the second kind disorder is defined by the ratio of the half width of the distribution function, Δa to the lattice constant a. The ratio $\Delta a/a$ is defined as g. The g value is reflected in the line width Δb as $\Delta b^2 = (1/2a)^2(4\pi^4 g^4 h^4 + C)$, where h is the reflection order and C is the constant term [28]. Thus value g can be obtained by the plot of the square of the integral width against the 4th power of reflection order. Examples are shown in the inset of Fig. 4 for the case of 4h and 8h iodinated PM. The slope of the regression line of the figure gives the g value as 0.035 for 8h iodinated PM. The same analysis was performed for each iodinated PM and as the result, the g value varied from 0.012 to 0.037.

3.3 The relation between the second kind of disorder and the absorption maximum of iodinated PM

The change of g value against iodination time which is obtained for one series of experiments is shown in Fig. 4. The finite value (0.017) for native PM is due to the geometry of experiment other than disorder. The broadening by the reason other than crystalline disorder is equally contributed to every iodinated PM. Therefore the change from the native PM's value is only meaningful. From Fig. 4, the second kind of disorder becomes prominent beyond 1h of iodination time and grows rapidly. This is already implied by Fig. 2. The spectral change of this series of experiments is shown in Fig. 5. The behavior of the change of g value shows good agreement with the shift of the wavelength of the absorption maximum.

The spectrum and the g values were measured and calculated for various iodinated PM samples. The g value is plotted against λ_{max} in cm^{-1} in Fig. 6. It is clear that both are well correlated. The λ_{max} reflects the

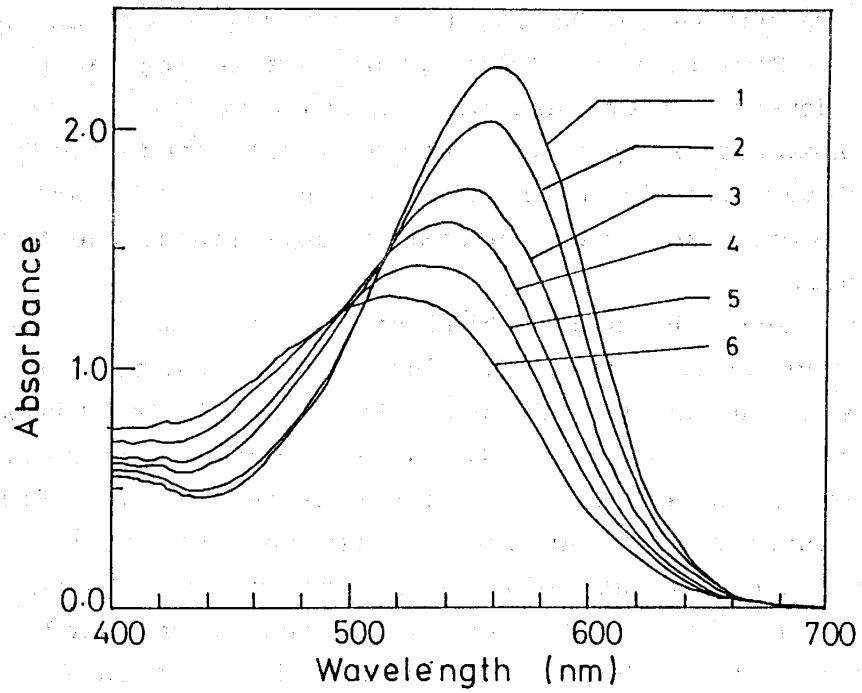

Fig. 5 The change of absorption spectrum in visible region during iodination. Note the blue shift of the absorption maximum and the decrease of absorbance. A similar spectrum was reported by Scherrer et al. [19]. 1. native PM, 2. 40 min-, 3. 90 min-, 4. 2 h-, 5. 4 h- and 6. 8 h-iodinated PM, respectively which are corresponding to the PM with 0, 1, 3, 5, 6 and more than 6 iodine atoms per bR molecule. Samples were the same as ones used for Fig. 4.

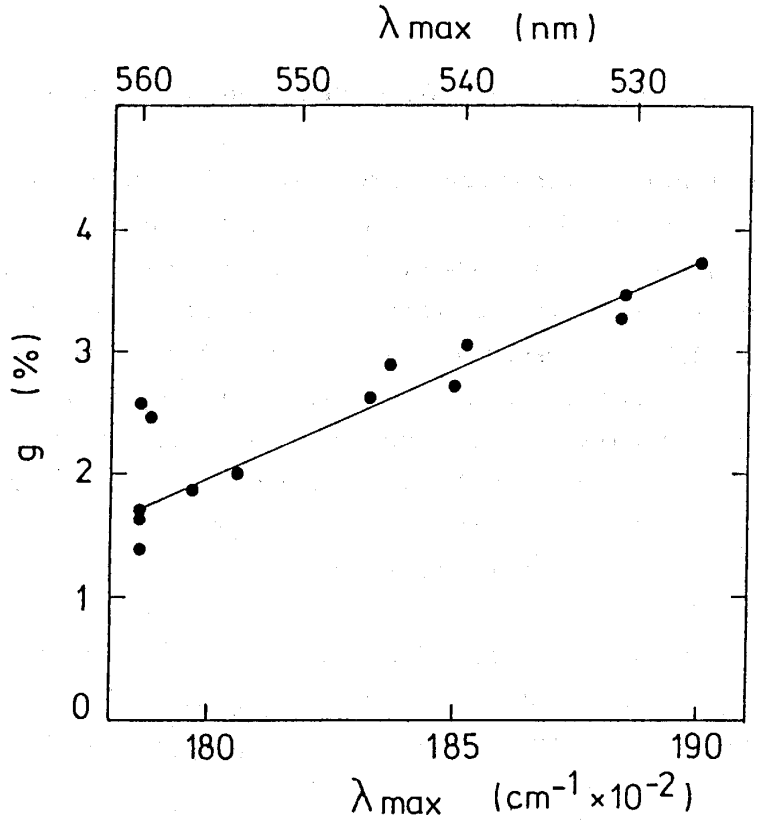

Fig. 6 The correlation between the g value and the wavelength at absorption maximum in cm^{-1}. The local change around retinal is reflected in the disorder of the crystalline structure.

environment of retinal, in the other word, the local conformational change of bR. On the other hand, the g value reflects the bulk structure of PM, that is, the change of inter-trimer interaction. Figure 6 indicates that the change in local structure affects the crystalline structure.

4. Discussion

4.1 Analysis of reflection width

As mentioned above, the disorder which causes the broadening of the reflection width is the second kind. A decrease of the size in the crystalline region also causes a broadening of the line width. In this case, however, the line width is independent of the scattering angle. As is clear from Fig. 2, the line widths are broader for the higher reflections, indicating that the broadening of the line widths observed for the iodinated PM originates from the second kind of disorder but not a decrease of the crystalline size.

Assuming that the broadening is due to the decrease of crystalline size, the size of crystalline region is estimated to be several hundreds Å from the line width, even for the early stage of iodinated PM. Freeze-fracture electron micrograph, however, shows the PM with a diameter of about 5000Å [29]. The discrepancy indicates that the broadening of line width is not explained by the decrease of the crystalline size but by the disorder. Thus, the above assumption is not valid.

4.2 Analysis of the second kind of disorder

In the general case of three dimensional crystal, the analysis shown in Fig. 4 should be performed with the use of the reflections of (nh, nk, nl) (h, k, l are fixed and n=1,2,3...) [28]. In the present case, we cannot apply this principle. We recorded the reflection up to (3,2). The reflections which allow the above criterion are (1,0), (2,0), (3,0) and (4,0). Among these four reflections, (1,0) is blurred by the reflection from the transverse structure of PM, and (3,0) cannot be observed clearly in every case because (3,0) is an intrinsically weak reflection. Thus, in order to obtain Fig. 5, we used every reflection whose order regards as $\sqrt{h^2+k^2+hk}$. This expansion would be valid only when the crystal is isotropic and the second kind of disorder is also isotropic. The isotropic disorder means that the distribution function H(x) is the same for all principal axes and is a function of the distance from the lattice point. The space group P3 for PM [3,5,6] satisfies the former assumption that the crystal is isotropic. The fact that the trimer of bR can be regarded as a cylinder at low resolution [3] may allow the latter assumption. Finally, the plot in Fig. 4 can be well approximated by a

line, indicating these assumptions are not far valid.

4.3 The second kind of disorder and M intermediate

The M intermediate is stabilized by iodination [19,20], its formation rate increases and its decay rate decreased [19]. Especially, the growth of the second kind of disorder is closely related to the decrease of the decay rate of M intermediate. The half time of M decay was plotted against iodination time by Scherrer et al who performed iodination under the same conditions as ours [19]. The behavior shows quite good agreement with the change of g value shown in Fig. 5.

The interaction between retinal and tyrosine residues may directly define the properties of iodinated PM [21]. Recent studies using the genetically modified PM strongly suggest that the tyrosine residue which involves in the photocycle is only the tyrosine 185 [30]. The tyrosine 185 is considered to be one of the most insensitive tyrosine residues to iodination judging from its location [31,32]. As one possibility, we consider that the disorder rather than the interaction between tyrosines and retinal is closely related to the stability of the M intermediate.

In order to understand why iodination causes the disorder, as well as to understand the properties of iodinated PM, it is essential to localize the iodine binding site. The small-angle X-ray scattering studies on solubilized bR revealed that some of the outer 4 helices were mainly iodinated near both surface [33].

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